



## **EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on the evaluation of the safety and efficacy of Listex<sup>TM</sup> P100 for the removal of *Listeria monocytogenes* surface contamination of raw fish**

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## SCIENTIFIC OPINION

### Scientific Opinion on the evaluation of the safety and efficacy of Listex™ P100 for the removal of *Listeria monocytogenes* surface contamination of raw fish<sup>1</sup>

EFSA Panel on Biological Hazards (BIOHAZ)<sup>2, 3</sup>

European Food Safety Authority (EFSA), Parma, Italy

This scientific output, published on 19 April 2012, replaces the earlier version published on 20 March 2012.\*

#### ABSTRACT

Studies evaluating the safety and efficacy of Listex™ P100 to reduce *Listeria monocytogenes* contamination on raw fish were assessed. The material should not present human toxicological problems because the bacteriophage P100, used as active principle, is not regarded as harmful to consumers nor to organisms other than *Listeria* spp., and because the fabrication parameters do not include anything obvious that might compromise safety. Data of studies considered indicate that Listex™ P100 is listericidal on inoculated catfish and salmon samples, but do not allow definitive conclusions on efficacy in reducing *L. monocytogenes* counts on raw fish nor on its impact on *L. monocytogenes* contamination levels in finished product. It was not possible to estimate the potential listeriosis risk reduction by treating raw fish with Listex™ P100. The data were not adequate to allow firm conclusions on persistence or activity of P100 in stored fish. The proposed use of Listex™ P100 is unlikely to result in emergence of reduced susceptibility to biocides and/or resistance to key therapeutic antimicrobials: however, this conclusion may need verification. No information was provided on survival of P100 in processing wastewater or the environment, or on the potential accumulation of naturally P100 resistant *L. monocytogenes* variants. Pilot and industrial scale studies should consider parameters affecting decontaminating efficacy, and should verify that application on raw fish has an impact on reduction of *L. monocytogenes* contamination on the final product. The persistence or activity of P100 as well as potential changes in *L. monocytogenes* counts should be evaluated during fish storage. Tests to investigate potential development of resistance or reduced susceptibility to biocides and key therapeutic

<sup>1</sup> On request from the European Commission, Question No EFSA-Q-2011-00959, adopted on 08 March 2012.

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antimicrobials, following use of Listex™ P100, are recommended. The continuous effectiveness of Listex™ P100 against *L. monocytogenes* and the potential for selection and dominance of strains naturally-resistant to P100 should be monitored.

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#### **KEY WORDS**

Decontamination, raw fish, Listex™ P100, efficacy, toxicological safety assessment, antimicrobial resistance, environmental impact

## SUMMARY

Following a request from the European Commission, the Panel on Biological Hazards (BIOHAZ Panel) was asked by the European Food Safety Authority (EFSA) to deliver a Scientific Opinion on an application dossier submitted by Microcos BV (the Netherlands) for the approval of Listex™ P100 for spraying or dipping uses aimed to reduce *Listeria monocytogenes* surface contamination of raw fish.

The Commission asked EFSA to issue a Scientific Opinion on the assessment of the safety and efficacy of Listex™ P100 when used to reduce *L. monocytogenes* surface contamination of raw fish. Specifically, the task was to consider the toxicological safety of the substance, its antimicrobial efficacy, the potential emergence of reduced microbial susceptibility to biocides and/or resistance to therapeutic antimicrobials linked to the use of the substance, and any risk related to the release of the processing plant effluents containing the substance into the environment. The assessment was based on the document “Guidelines on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption” published by EFSA<sup>4</sup>.

Concerning human toxicological safety, the Listex™ P100 preparation should not present problems because the bacteriophage P100 used as active principle is not regarded as harmful to consumers. Furthermore the Listex™ P100 manufacturing parameters do not include any obvious components or steps that might compromise safe use of the preparation.

The experiments of the two relevant for consideration laboratory studies used to assess the efficacy of Listex™ P100 as a decontaminating agent for raw fish were classified as of ‘low strength of evidence’. Thus their data are indicative of a disinfectant effect that may be reproducible in practice but do not allow definitive conclusions on the efficacy of the product in reducing naturally occurring low *L. monocytogenes* counts on raw fish. Results were not validated under pilot plant or industrial conditions. Data presented indicated that Listex™ P100 is listericidal. Reductions were in the range of 1.4 to 3.5 log<sub>10</sub> cfu/g on inoculated fresh catfish and salmon fillet samples. Limitations of the studies were that only two fish types were tested and sample sizes were generally small and with few repetitions. Additionally only a two-strain, two-serotype mixture of *L. monocytogenes* was evaluated, no strains of fish origin were included, and lower *L. monocytogenes* contamination levels, usually expected on fish, were not tested.

*Listeria monocytogenes* counts showed an upward trend in treated catfish fillets during the 10-day storage at 4 °C or 10 °C, but not in treated salmon fillets stored for 10 days at 4 °C. It is unclear whether these counts were due to total prevention of bacterial growth or to the balance between growth and death of bacterial cells. Bacteriophage P100 titre remained stable on raw salmon fillet samples for 4-7 days at 4 °C, while it decreased during the first 4 days of the 10-day storage period, at 4 °C and 10 °C, of treated catfish samples. Thus, the data provided were not adequate to draw firm conclusions on persistence or activity of P100 during the 10-day storage of treated fish samples.

No evidence was provided to demonstrate the impact of treating raw fish on *L. monocytogenes* contamination levels in the finished product. Overall, the evidence provided is not adequate to evaluate potential human listeriosis risk reduction by treating raw fish with Listex™ P100.

Although the experimental evidence available is very limited, it is considered unlikely that the proposed use of Listex™ P100 might result in the emergence of reduced susceptibility to biocides and/or resistance to key therapeutic antimicrobials. Additionally, testing should

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<sup>4</sup> EFSA Journal 2010;8(4):1544

monitor the potential for selection and dominance of *L. monocytogenes* strains naturally-resistant to P100.

No information was provided on the survival of P100 in processing wastewater or the environment in order to address potential risks related to the release of the processing plant effluents, linked to the use of the substance, into the environment.

Considering the limitations of the proof of concept investigations evaluated by the Panel, it is recommended that pilot and industrial scale studies should address parameters such as types and size of fish, stage of processing for application of Listex™ P100, multiple and more appropriate strains used for inoculation, contamination levels comparable to those naturally present, fish sample size and repetitions. Verification is needed that treatment of raw fish with Listex™ P100 will have an impact on *L. monocytogenes* contamination levels in the final fish product. The persistence or activity of P100 as well as potential changes in *L. monocytogenes* counts should be further evaluated during storage of treated fish samples. The continuous effectiveness of Listex™ P100 against *L. monocytogenes* and the potential for selection and dominance of strains naturally resistant to P100 should be monitored. This additional knowledge should be provided in order to understand the influence of variables associated with practical applications, and, thus, allow commercial operators to select conditions of use that are validated and verified according to HACCP principles.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The EU food hygiene legislation is aimed at protecting consumers against potential risks to health and maintaining a high level of consumer protection at all stages of the food chain. This objective must be achieved by applying the appropriate measures, including good hygiene practices and hazard control measures at each step of the food chain.

According to EU scientific advice<sup>5</sup>, decontamination practices can constitute a useful tool in further reducing the number of pathogenic microorganisms but the use of substances intended to remove microbial surface contamination should only be permitted if a fully integrated control programme is applied throughout the entire food chain. Those substances shall be assessed thoroughly before their use is authorised.

Article 3 (2) of Regulation (EC) No 853/2004 provides a legal basis to approve, and therefore authorise, the use of substances other than potable water to remove surface contamination from products of animal origin.

In addition to the safety of the substance, a matter of concern is also the potential emergence of reduced susceptibility to biocides and/or the resistance to therapeutic antimicrobials and the impact of the substance or its by-products on the environment.

Therefore, before taking any risk management decision on their approval, a risk analysis should be carried out taking into account the results of a risk assessment based on the available scientific evidence and undertaken in an independent, objective and transparent manner, other legitimate factors, and the precautionary principle.

## EFSA GUIDANCE AS PROVIDED BY THE EUROPEAN COMMISSION

On 14 April 2010, the European Food Safety Authority (EFSA) issued a revision of a guidance document<sup>6</sup> on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption.

## APPLICATION FOR APPROVAL AS PROVIDED BY THE EUROPEAN COMMISSION

On 15 July 2011, the Commission received an application dossier from Microcos BV (the Netherlands) for the approval of Listex™ P100 to remove *Listeria monocytogenes* surface contamination of raw fish. The dossier is enclosed to this request.

## TERMS OF REFERENCE AS PROVIDED BY EUROPEAN COMMISSION

EFSA is requested to evaluate the safety and efficacy of Listex™ P100 to remove *Listeria monocytogenes* surface contamination of raw fish, considering:

1. the toxicological safety of the substance;
2. the efficacy, i.e. does the use of the substance significantly reduce the level of contamination of *Listeria monocytogenes*;
3. the potential emergence of reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials linked to the use of the substance; and

<sup>5</sup> SCVPH (Scientific Committee On Veterinary Measures Relating To Public Health), 1998. Report on the benefits and limitations of antimicrobial treatments for poultry carcasses, 30 October 1998. SCVPH (2003) Opinion on the evaluation of antimicrobial treatments for poultry carcasses ([http://ec.europa.eu/food/fs/sc/scv/out14\\_en.pdf](http://ec.europa.eu/food/fs/sc/scv/out14_en.pdf)).

<sup>6</sup> EFSA Journal 2010;8(4):1544.

4. the risk related to the release of the processing plant effluents, linked to the use of the substance, into the environment.

Clarification of the terms of reference:

Following discussion with the Commission services, it was clarified that the following should also be considered in the Scientific Opinion:

1. treatment of only raw fish as the initial material for the production of a variety of products, and
2. persistence of bacteriophages (i.e. continual functioning) in the food.

**APPROACH TAKEN TO ANSWER THE TERMS OF REFERENCE**

After having received this request from the European Commission, the European Food Safety Authority (EFSA) assigned the mandate to the Panel on Biological Hazards (BIOHAZ Panel) for assessment. The terminology and procedure used by the Panel in this assessment conform with the “Guidelines on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin intended for human consumption” prepared by EFSA (EFSA Panel on Biological Hazards (BIOHAZ), 2010).

The BIOHAZ Panel adopted all chapters and the respective conclusions of this Opinion on 8 March 2012.



## ASSESSMENT

### 1. Introduction

As indicated in the application dossier, the purpose of the proposed treatment is “to eradicate or decrease *Listeria monocytogenes* present on raw fish”. The formulated product has the trade name Listex™ P100 and contains bacteriophage P100 at a concentration of  $2 \times 10^{11}$  plaque-forming units (pfu) per ml. Approval was sought for spraying or dipping treatments of raw fish using up to  $1 \times 10^9$  pfu of P100 per gram of product. As stated in the application dossier, the concentration to be used depends on the log unit reduction desired as well as other factors.

The aim of the present Opinion is to assess the safety and efficacy of Listex™ P100 to reduce *L. monocytogenes* surface contamination on raw fish considering (1) the toxicological safety of the substance, (2) the efficacy, i.e. does the use of the substance significantly reduce the level of contamination of *L. monocytogenes*, (3) the potential emergence of reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials linked to the use of the substance, and (4) the risk related to the release of the processing plant effluents, linked to the use of the substance, into the environment. Each of these assessments is described subsequently.

### 2. Product characteristics and conditions of application

According to the application dossier, Listex™ P100 is a water formulated product that contains bacteriophage P100 at a concentration of  $2 \times 10^{11}$  pfu/ml; the molecular weight of one particle of P100 is approximately  $1.2 \times 10^8$  Dalton; and its size is approximately 300 nm. According to Klumpp et al. (2008) the size of bacteriophage P100 is, head diameter 89.55 nm, tail length 198.24 nm, tail diameter 19.0 nm.

Production of Listex™ P100 is based on a fermentation process using non-pathogenic *Listeria innocua* (Buchrieser et al., 2003) followed by several filtration steps with the objective of removing the propagation bacteria and cell debris. To ensure sterility, the final product is evaluated by testing for presence of bacteria, yeasts and moulds. The shelf life of Listex™ P100 is considered to be six months.

The application dossier states that Listex™ P100 is not chemically reactive under the conditions of the intended use. Bacteriophages consist of a DNA core that is surrounded by a protein shell. Therefore, their breakdown products consist of amino acids and nucleic acids, which, as stated by the applicant, are already present in abundance in foods of animal origin.

As indicated in the application dossier, use conditions selected for Listex™ P100 to be effective may vary within the following limits, which, as noted by the BIOHAZ Panel, are within those of *L. monocytogenes* growth:

- temperature: optimum 30 °C, range 1 to 35 °C;
- pH: optimum 7.7; range 5.5-9.5;
- water activity: minimum 0.92, optimum 0.99; and
- Sodium Chloride (NaCl): high tolerance up to saturated solutions.

According to the application dossier, and as further clarified by the applicant, the optimal use of Listex™ P100 is dependent upon the specific circumstances of any given situation as in practice most fish processing units operate with different raw materials, processing steps, processing conditions, and processing times. Specifically, the applicant provided the following clarification: These “activity parameters are broad, and meant only to ensure that Listex™ P100 is used under circumstances where the product will function properly. In practice, most fish processing units operate with different raw materials, processing steps, processing conditions and processing times. The optimal application process is therefore dependent upon the specific circumstances of each situation. For raw fish fillets, for example, the best time to treat is just after filleting, but with the skin still on”. The applicant also

states that “in all cases the intended time-point of treatment is as early in the process as feasible, when and where the *L. monocytogenes* are most susceptible to bacteriophages, and to ensure that contaminations are not spread throughout the processing environment and thus become the source of recontamination at a later point in time.”

The use of Listex™ P100 is recommended by the applicant to be either by spraying or dipping in a manner that distributes bacteriophages evenly over the treated surface. A manufacturer might normally use a dipping process applied for 1 minute. Once Listex™ P100 is applied in the right dose to the surface of the product, a time interval of approximately 30 minutes is needed for the bacteriophages to find and kill *Listeria* host cells.

According to the application dossier, the concentration that is used by the fish processor depends on the log units of pathogen reduction desired. In general, the amount of bacteriophage applied should be adequate and the exposure time long enough for contact with bacterial cells to occur through diffusion, as bacteriophages are non-motile. Overall the concentration used does not exceed  $1 \times 10^9$  pfu/g. Since Listex™ P100 is applied by spraying or dipping, the activity, survival and inactivation parameters of the Listex™ P100 solution should be considered.

The concentration of bacteriophages required to achieve the desired pathogen reduction varies with different foods as the bacteriophages also interact with the food matrix decreasing the amount of and the time in which this occurs to varying degrees. An approximate 2-log unit reduction of *L. monocytogenes* on fish filets can typically be achieved with  $2 \times 10^7$  bacteriophage per square centimetre as long as contact time exceeds 30 minutes.

There is no step for the neutralization, removal or recycling of Listex™ P100 because, according to the application dossier, “there is no: (i) health risk relating to the presence of ‘residues’ (toxicological safety); and (ii) technological function in the end product.” According to the application dossier, “removal of bacteriophages is not necessary because bacteriophages are only active for a limited amount of time, their breakdown products are the same as substances already present in food, and lytic bacteriophages are not regarded as a health hazard for humans”.

The treated raw fish are mainly destined for use in the production of cold-smoked and cured fish products such as gravlax and cured herring. Also raw fish that will ultimately be cooked before consumption is to be considered, for reasons of preventing cross-contamination or product abuse downstream of the primary processing steps.

The applicant further states in the application dossier, that Listex™ P100 should be used as an additional risk-reduction measure. There is no standard guarantee that a specific reduction level can be achieved through a standard application, and use of the product does not change the manufacturer’s obligations to test end-product safety for product release and certainly not for a lack of proper hygiene. It is further stated that regular or intensified testing for *L. monocytogenes* pre- and after-treatment should confirm the results of the Listex™ P100 application.

### **3. Public health concerns associated with listeriosis and seafood**

The BIOHAZ Panel has summarized knowledge on the pathogen and the infection through consumption of contaminated seafood (presented in Appendix A).

### **4. Biology of bacteriophages and use as control agents in food production**

The BIOHAZ Panel, based on the previous EFSA Scientific Opinion on “The use and mode of action of bacteriophages in food production” (EFSA, 2009), has also summarized material related to the biology and use of bacteriophages as antibacterial agents (Appendix B). Related material was also extracted from the application dossier. The specific characteristics of P100 are described in other sections.

## 5. Methods of analysis used by the applicant

According to the application dossier, the analytical methods to detect P100 are a polymerase chain reaction (PCR) and a plaque assay. The latter is the standard method to detect and quantify bacteriophages. In short, it involves preparation of different dilutions of bacteriophage stock and inoculation on susceptible (*Listeria* spp.) lawns. After incubation, plaques formed by individual bacteriophages are counted allowing calculation of their concentration per ml.

The method used for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp., as included in Annex 9 of the application dossier, is the UK Health Protection Agency (HPA) Standard Method (HPA, 2009).

## 6. The toxicological safety of the substance to humans

### 6.1. Evaluation

Three types of parameters were taken into account in evaluating the safety/toxicity of Listex™ P100 application for *Listeria* decontamination of raw fish: the potential for temperate bacteriophage presence in the *L. innocua* propagation strain; the bacteriophage P100 itself; the ingredients and mode of fabrication of the preparation to be applied and their possible effects on consumers of fish.

#### 6.1.1. Potential presence of temperate bacteriophages in the Listex™ P100 preparation

The potential for presence of temperate bacteriophages in the *L. innocua* propagating strain and their transfer in the Listex P100 preparation was not addressed in the application dossier. Also, there is no sufficient knowledge of the P100 preparation process to comment meaningfully on the possibility of temperate phage 'carry-over'. However, if such an event had occurred, then the two types of bacteriophages (i.e. lytic and temperate) should have been phenotypically evident on plates used for calculation of concentration.

Although potentially an issue in certain circumstances, 'carry-over' of virulence genes by temperate bacteriophages should not be of concern in the production process of Listex™ P100 because the bacteriophage propagating organism (*L. innocua*) is not pathogenic and is lacking the virulence gene cluster (vgc) of *L. monocytogenes* (Chakraborty et al., 2000). This implies that even if the *L. innocua* genome contained resident prophages, they would not carry any virulence determinants. In addition, temperate bacteriophages usually have narrow host ranges. Thus, even if a *L. innocua* temperate phage contaminated the Listex™ P100 preparations it should not be infective to *L. monocytogenes*. Furthermore, as stated in the application dossier, a routine procedure to check bacteriophage P100 purity is gel electrophoresis of its DNA. If other bacteriophages were present in sufficient concentration, their DNA should be detected by this method.

#### 6.1.2. The bacteriophage P100

Bacteriophage P100 was isolated from sewage of a dairy plant on the basis of its ability to lyse *L. monocytogenes*. It belongs to the family *Myoviridae*, composed of bacteriophages that present contractile tails to inject their genomic double stranded DNA molecule into the bacterial hosts. In the 131 kbp genome of P100, 174 open reading frames encoding for proteins, plus 18 tRNA determinants have been mapped (Gen Bank reference: DQ004855) (Carlton et al., 2005). Functions could only be ascribed to 25 of the putative proteins, while most of them present diverse degrees of homology to those of other bacteriophages such as K, A511, LP65 and SPO1, which infect strains of *Staphylococcus*, *Listeria*, *Lactobacillus* and *Bacillus*, respectively. In addition to this, all of them share their morphology, infect low G+C Gram-positive bacteria, are strictly virulent, affect wide spectra of susceptible bacterial strains, and, in the cases where this characteristic is known, have terminally redundant, non-permuted genomes, all of which justified their inclusion into the SPO1-like group of bacteriophages and the proposed subfamily *Spounavirinae* (Klumpp et al., 2008; Klumpp et al., 2010).

Most of these characteristics (including those outlined in section c of Appendix B) are relevant to the safe use of bacteriophage P100 for control of *Listeria* fish-contamination (EFSA Panel on Biological Hazards (BIOHAZ), 2009), as follows:

- exclusively virulent: this eliminates the possibility of generating lysogenic hosts that, by definition, would be immune to superinfection by the same virus and that might acquire new, bacteriophage encoded, properties (lysogenic conversion);
- one unit genome packaging: the strict recognition requirement of the DNA sequence termini by the bacteriophage terminase will preclude the formation of transducing bacteriophages and thus avoid bacterial DNA transfer into their capsids; and
- broad host range restricted to *Listeria*: Carlton et al. (2005) indicated that over 95 % of approximately 250 different *Listeria* isolates tested including diverse serovars of *L. monocytogenes* tested were susceptible to P100. No other bacterial genera are affected, thus ensuring containment of the treatment (Carlton et al., 2005). In another study (OFIMER, 2011) using an in vitro test, 78 % of the 42 *L. monocytogenes* strains isolated from smoked salmon and smoked trout, were sensitive to Listex™ P100 tested, while 12 % were classified as intermediary, and 10 % as resistant.

### 6.1.3. The decontaminating formulation

This part is based on the information contained in a technical report provided by the applicant.

As indicated, P100 is propagated on a strain of non-pathogenic *L. innocua*. The growth medium for the host is composed of non-animal ingredients. The applicant provided specifications for the ingredients and demonstrated that toxicological relevant impurities were not present. Medium sterilization is by filtration, thus avoiding the possible generation of toxic compounds driven by high temperature.

Downstream processing is essentially performed by successive filtrations to separate bacteriophages from unlysed bacteria and other debris, for bacteriophage concentration, and for purification of the suspensions; the final bacteriophage concentration is adjusted with water and the product is stored at 4 °C. Adequate controls are performed for microbial contamination and potency of the preparation (through titration of viable virions). Finally, the applicant states in the dossier that a HACCP plan and Good Laboratory Practices (GLP) are in operation during production.

### 6.1.4. The potential effect of bacteriophage P100 on consumer's health

As is indicated in Appendix B, bacteriophages are part of the indigenous microbiota of eukaryotic organisms, including humans. Bacteriophages are routinely consumed in fermented foods.

Upon consumption, the bacteriophages numbers should be reduced substantially because they are subjected to severely deleterious conditions, such as the stomach acidity and the attack by digestive proteinases including pepsin and trypsin (the capsid is made out of protein). Furthermore, commercial use of phage-based preparations has been granted by the US Food and Drug Administration (US FDA) for use on ready-to-eat (RTE) foods, which are considered to be GRAS (Generally Regarded As Safe) in the USA. Among them is Listex P100 itself and a range of other preparations such as LMP-102, also active against *L. monocytogenes*, Agriphage, used against tomato and pepper spot and even anti-*Escherichia coli* and anti-*Salmonella* washes for live animals prior to slaughter (Coffey et al., 2010; Hagens and Offerhaus, 2008).

Furthermore, the raw fish treated with P100 should in most cases be processed before consumption. Processing, such as cooking, should inactivate the bacteriophage because bacteriophages do not survive when exposed for a few minutes to temperatures above 70 °C. Additional processes such as marinating, curing and freezing may also be deleterious to bacteriophages. It needs to be noted that in some cases fish may also be consumed raw (e.g. sushi).

### 6.1.5. Possible reserves

- Bacteriophage P100 does not harbour any gene whose product is homologous to bacterial toxins or other virulence factors; this should serve as proof of safe application. It should be noted, however, a majority of the open reading frames of P100 encode proteins with no matches in relevant databases. This may indicate the possibility of a toxinogenic potential. In fact, protein gp71 of the bacteriophage appears to present in its C-terminal end short stretches which are similar to the allergenic protein  $\gamma$ -gliadin (Carlton et al., 2005). A consultation with the allergy expert of the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) led to the conclusion that “gp71 is unlikely to trigger an allergic reaction in wheat sensitive individuals even if it were equal in allergenic potency to the wheat protein gamma-gliadin, given the small amounts of phage protein that is predicted to be present on the fish even when the ‘worst case scenario’ is contemplated”. In addition, as described by Carlton et al. (2005), an oral toxicity study has been performed on ten rats of about 8 weeks of age. The authors claim that feeding these rats with  $5 \times 10^{11}$  bacteriophages/day for five consecutive days did not reveal any alterations in their survival, body weight, behaviour, or appearance and the necropsy results were normal. This study was performed according to OECD GLP principles.
- It is not clear whether the Listex™ P100 phage-manufacturing procedures will eliminate cell debris such as host cell wall fragments. These, and especially the teichoic acid/lipoteichoic acid fractions, might interact with toll receptors on the surface of the myeloid cells and act as immune adjuvants, as might do the P100-lysed contaminating *Listeria*. According to the NDA Panel expert, this should be no cause for concern. Reasons for this include the expected very low degree of contamination on fish and its treatment before any substantial bacterial growth. Additional considerations indicated by the NDA Panel expert are the large amounts of bacterial debris present in some foods (e.g. some fermented foods), and the vast number of microbes residing in the gastro-intestinal tract.

### 6.2. Conclusions

The Listex™ P100 preparation should not pose a risk to human health because: bacteriophage P100 fulfills the safety requirements included in the QPS Opinion (EFSA Panel on Biological Hazards (BIOHAZ), 2009); the remnants of the digested bacteriophage are of no health concern; the chemicals and other parameters used in its manufacture do not include any obvious components or steps that might compromise safe use. Two possible reserves considered should not be relevant for the uses and conditions sought in the application. They are the lack of putative function ascribable to most of its proteins and the lack of a specific control for presence of *Listeria* cell wall fragments in the preparation.

## 7. The efficacy, i.e. does the use of the substance significantly reduce the level of contamination of pathogenic microorganisms

### 7.1. Introduction

In order to assist in assessing the efficacy of a decontaminating agent, EFSA issued in 2010 a revised guidance document (EFSA Panel on Biological Hazards (BIOHAZ), 2010) which points out the major components and data that an application dossier should contain in order to demonstrate that the substance intended to be used for the reduction of microbial surface contamination of foods of animal origin is efficacious. These guidelines have been used in this assessment of Listex™ P100 for use in the decontamination of raw fish relative to *L. monocytogenes*.

According to the EFSA guidance document, the use of substance(s) as decontaminating treatments will be regarded efficacious when any reduction of the prevalence and/or numbers of pathogenic target microorganisms is statistically significant as compared to the control (e.g. water) and, at the same time, this reduction has a positive impact on reduction of human illness cases. Risk assessment studies on other microbial species (EFSA Panel on Biological Hazards (BIOHAZ), 2011a, 2011b) have shown



that even 0.5 log<sub>10</sub> unit microbial reductions may reduce consumer risks to a significant extent. In addition, there is a linear correlation between reductions in prevalence and reductions of consumer risks. Efficacy depends on a range of factors such as concentration of the decontaminating agent, the microbial pathogen and its load of the surface, contact time, temperature, mode of application, and other conditions of application.

## 7.2. Selection of studies for evaluation of efficacy

The application dossier summarizes the data from five peer-reviewed published papers and one project report examining the efficacy of Listex™ P100 on various food products. The applicant seeks approval for treatments of raw fish using up to 10<sup>9</sup> pfu/g of food product as early in the process as feasible and applied either by spraying or dipping, with broad limits of conditions (temperature, pH, water activity and NaCl level) as specified above.

The body of evidence from the studies submitted in the application dossier was evaluated by the EFSA BIOHAZ Panel, taking into account whether the studies were done in the laboratory, a pilot plant or a processing plant, and whether they used inoculated or naturally contaminated fish. Table 1 presents how combinations of industrial-, pilot- or laboratory-scale study settings and evaluation of natural or inoculated contamination were used to classify the strength of evidence of the data in each study. These criteria were originally presented in the FAO/WHO report on Benefits and Risks of the Use of Chlorine-containing Disinfectants in Food Production and Food Processing (FAO/WHO, 2008), and were adapted from a previous EFSA Opinion (EFSA Panel on Biological Hazards (BIOHAZ), 2011b).

**Table 1:** Relative strength of the contribution of study data to the general body of evidence, based on study type (based on EFSA Panel on Biological Hazards (BIOHAZ) (2011b))

Study type	Natural contamination	Inoculated studies
Industrial	High	Not applicable
Pilot-scale <sup>a</sup>	High <sup>b</sup> /medium	Medium <sup>c</sup>
Laboratory	Medium <sup>c</sup>	Low <sup>d</sup>

a Experiments using industrial equipment in non-industrial settings.

b If the pilot process is representative of the industrial process; otherwise, evidence makes a ‘medium’ contribution to the body of evidence.

c Data would not be sufficient to inform a quantitative microbial risk assessment or to allow definitive conclusions on risk reduction.

d Data are indicative of a disinfectant effect that may be reproducible in practice, but individually do not allow definitive conclusions on risk reduction.

Of the six papers submitted for consideration, four were excluded from the evaluation because the studies described were outside the scope for which the applicant is seeking approval. More specifically, these four papers were excluded because food products other than raw fish were examined (Table 2).

**Table 2:** The six papers submitted by the applicant and the reasons for inclusion/exclusion of papers in/from the assessment

Reference	Inclusion in assessment	Reason for exclusion	Industrial /pilot/lab	Natural /inoculated	Microorganisms	Product treated	Strength of evidence
Soni et al. (2010)	YES		Lab	Inoculated	<i>L. monocytogenes</i>	Fresh channel catfish filet	Low
Soni and Nannapaneni (2010)	YES		Lab	Inoculated	<i>L. monocytogenes</i>	Raw salmon filet	Low
OFIMER (2011)	NO	Raw fish not used <sup>a</sup>					
Guenther et al. (2009)	NO	Raw fish not used <sup>b</sup>					
Holck and Berg (2009)	NO	Raw fish not used <sup>c</sup>					
Carlton et al. (2005)	NO	Raw fish not used <sup>d</sup>					

a Smoked salmon used.

b Hot dogs, sliced turkey meat, smoked salmon, seafood (cooked and chilled cocktail of shrimp, mussels and calamari), sliced cabbage, and lettuce leaves used.

c Sliced cooked ham used.

d Surface-ripened red-smear soft cheese used.

The assessment of the efficacy of Listex™ P100 to remove *L. monocytogenes* surface contamination of raw fish was therefore based on two of the six papers included in the application dossier. The papers included in the evaluation described studies with low strength of evidence and using inoculated fresh channel catfish (Soni et al., 2010) and raw salmon (Soni and Nannapaneni, 2010) fillet samples.

### 7.3. Evaluation of studies on efficacy

The influence of bacteriophage dose, bacteriophage contact time, and storage temperature on the listericidal activity of P100 in reducing the inoculated *L. monocytogenes* loads on the surface of fresh channel catfish fillets is described in Soni et al. (2010). More specifically, according to Soni et al. (2010), the P100 stock solution of approximately  $10^{11}$  pfu/ml in buffered saline, as determined by plaque formation assay, was serially diluted in physiological saline to prepare the desired application concentrations. Duplicate 5 g samples of fresh catfish (approximately 2 cm<sup>2</sup> top surface area) were inoculated with 50 µl of a two-strain mixture (strains, EGD (BUG 600; serotype 1/2a) and strain Scott A (serotype 4b)) of *L. monocytogenes* suspension to yield an inoculation level of approximately 4.3 log<sub>10</sub> cfu/g. The inoculum was uniformly spread on the white flesh fillet surface side by random spotting at five points of 10 µl each on the flesh side. The inoculum was air-dried for 15 min before treatment. In addition, large fillets (approximately 180–200 g) were also inoculated with 2 ml of the serially diluted two-strain mixture of *L. monocytogenes* and air-dried for 15 min to yield *L. monocytogenes* inoculation levels of about 4 log<sub>10</sub> cfu/g. After inoculation, the 5 g catfish fillet samples were surface treated with 100 µl of P100 applied at concentrations of 10<sup>9</sup>, 10<sup>7</sup>, or 10<sup>5</sup> pfu/ml to yield final application doses of 2×10<sup>7</sup> pfu/g (7.3 log<sub>10</sub> pfu/g), 2×10<sup>5</sup> pfu/g (5.3 log<sub>10</sub> pfu/g), or 2×10<sup>3</sup> pfu/g (3.3 log<sub>10</sub> pfu/g), respectively. For the no bacteriophage control, the fillet pieces were surface treated with 100 µl saline. Duplicate fillet samples in a weighing dish were sealed in Ziploc bags (16.5×14.9 cm) and incubated at 4 °C, 10 °C, or 22 °C. After incubation for 15 min, 30 min, 1 h, 2 h, 1 day, 4 days, 7 days, or 10 days, the fillet samples were subjected to *L. monocytogenes* enumeration. All experiments were repeated three times with two replications. Each large fillet was sprayed with 7.5 ml of Listex™ P100 suspension of 10<sup>9</sup> pfu/ml evenly on the entire white-flesh side using a hand-held spray bottle (2 oz mini fingertip sprayer). Untreated control fillets were sprayed with the same amount of saline. After 30 min or 2 h contact time with Listex™ P100 at 22 °C, fillets were enumerated for *L. monocytogenes*. Experiments were repeated twice.

According to the results, the *L. monocytogenes* reduction by P100 on fresh catfish fillet samples (5 g) was influenced by bacteriophage contact time and bacteriophage dose regardless of higher or lower temperature regimes tested. The reduction in *L. monocytogenes* loads ( $p < 0.05$ ) with the P100 dose of  $7.3 \log_{10}$  pfu/g was  $1.4\text{--}2.0 \log_{10}$  cfu/g at  $4^\circ\text{C}$ ,  $1.7\text{--}2.1 \log_{10}$  cfu/g at  $10^\circ\text{C}$ , and  $1.6\text{--}2.3 \log_{10}$  cfu/g at  $22^\circ\text{C}$  on raw catfish fillets. The levels of  $10^3$  and  $10^5$  pfu/g had no or minor effect on *L. monocytogenes*. The bacteriophage contact time of 30 min was adequate to yield  $1.3\text{--}1.6 \log_{10}$  cfu/g reduction in *L. monocytogenes*, whereas 15 min contact time with bacteriophage yielded less than  $1.0 \log_{10}$  cfu/g reduction in *L. monocytogenes* loads on catfish fillets. Maximum reductions at  $22^\circ\text{C}$  were achieved after 30 min of exposure, while no further reductions were detected after 30 min and up to 120 min. Reductions in large catfish fillets treated with  $7.3 \log_{10}$  pfu/g for 30 and 120 min at  $22^\circ\text{C}$  were  $1.7\text{--}2.3 \log_{10}$  cfu/g.

Surviving *L. monocytogenes* counts demonstrated an upward trend over the 10-day shelf life at  $4^\circ\text{C}$  or  $10^\circ\text{C}$ , while P100 titres on the catfish fillet samples, from the initial level of  $7.3 \log_{10}$  pfu/g, decreased to 5.5 and  $5.2 \log_{10}$  pfu/g during the 10-day shelf life at  $4^\circ\text{C}$  and  $10^\circ\text{C}$ , respectively. These results indicate a maximum loss of approximately  $1.8\text{--}2.1 \log_{10}$  pfu/g in P100 concentrations during 10 days of storage for fresh catfish fillets. It should be noted that reductions were mostly noticeable during the first 4 days of storage. These results would indicate that after addition to the food, bacteriophages become inactive, as indicated in the application dossier. According to the submitted dossier, active bacteriophage numbers decline from the moment of application due to various factors like adsorption of bacteriophages to particles, proteolytic degradation of the bacteriophage particle by chemicals and enzymes, temperature, salts and light (Garza and Suttle, 1998; Hurst et al., 1980; Suttle and Chen, 1992); eventually, bacteriophages fall apart into amino acids and nucleic acids.

The antilisterial activity of Listex™ P100 on the surface of raw salmon fillet tissue against *L. monocytogenes* serovars 1/2a and 4b was examined by Soni and Nannapaneni (2010). Fresh, whole raw salmon fillet tissue samples of approximately  $2\text{-cm}^2$  blocks (10 g) were prepared with the flesh side facing up. For evaluation of the effect of different P100 concentrations on *L. monocytogenes* reduction each raw salmon sample was inoculated with 50  $\mu\text{l}$  of a serially diluted, two-strain (serotype 1/2a and 4b) mixture to yield an inoculation level of approximately  $4 \log_{10}$  cfu/g. Samples were surface treated with serially diluted P100 on the flesh side by applying 100- $\mu\text{l}$  suspensions of  $10^{10}$ ,  $10^9$ ,  $10^8$ ,  $10^7$ , and  $10^6$  pfu/ml in physiological saline to yield final doses  $10^8$ ,  $10^7$ ,  $10^6$ ,  $10^5$  and  $10^4$  pfu/g, respectively. For the untreated control, each sample received 100  $\mu\text{l}$  of saline solution. The duplicate samples per treatment were placed in a polystyrene dish, sealed immediately in a Ziploc bag for incubation at  $4^\circ\text{C}$  for 2 h, and then enumerated for *L. monocytogenes*. To study the effect of P100 against low and high *L. monocytogenes* inoculum levels, serial dilutions of the pathogen cell suspension were spot inoculated at 50  $\mu\text{l}$  to yield 2, 3, or  $4 \log_{10}$  cfu/g on the flesh side of the 10-g raw salmon tissue sample. These tissue samples were then surface treated with P100 by adding 100  $\mu\text{l}$  of bacteriophage suspension to the flesh side, for a bacteriophage application dose of  $10^8$  pfu/g per 10 g of tissue sample. Each treatment, which consisted of duplicate tissue samples in a polystyrene dish, was immediately packed in a Ziploc bag for incubation at  $4^\circ\text{C}$  or  $22^\circ\text{C}$ , and then enumerated for *L. monocytogenes* after 30 min and 2 h. The effect of P100 on *L. monocytogenes* growth during the shelf life of raw salmon fillet tissue was examined by inoculating 10-g samples of raw salmon fillet tissue with approximately  $2 \log_{10}$  cfu/g of *L. monocytogenes* serotype mixture (1/2a and 4b) and treating the samples with P100 by applying 100- $\mu\text{l}$  of bacteriophage suspension to the flesh side to give a bacteriophage dose of  $10^8$  pfu/g. After treatment, the polystyrene dish containing duplicate tissue samples was immediately packed in a Ziploc bag for storage at  $4^\circ\text{C}$ , and analyzed for *L. monocytogenes* levels at 0, 1, 4, 7, and 10 days.

The results (Soni and Nannapaneni, 2010) indicated that on raw salmon fillet tissue, a bacteriophage concentration of  $10^8$  pfu/g was required to yield 1.8-, 2.5-, and  $3.5\text{-}\log_{10}$  cfu/g reductions of *L. monocytogenes* from its initial loads of 2, 3, and  $4 \log_{10}$  cfu/g at  $4^\circ\text{C}$  or  $22^\circ\text{C}$ . From the initial load of  $1.6 \log_{10}$  cfu/g, over the 10 days of storage at  $4^\circ\text{C}$ , *L. monocytogenes* growth was limited on the treated raw salmon fillet tissue to as low as  $0.3 \log_{10}$  cfu/g versus normal growth of  $2.6 \log_{10}$  cfu/g in the absence of bacteriophage. Bacteriophage P100 remained stable on the raw salmon fillet tissue over



a 10-day storage period; with only a marginal loss of 0.6 log pfu/g from an initial bacteriophage treatment of 8 log<sub>10</sub> pfu/g during the last days of storage. This is in contrast to the results of Soni et al. (2010) with catfish fillets summarized above.

In general, as indicated in the application dossier, the two papers of Soni et al. (2010) and Soni and Nannapaneni (2010), tested P100 efficacy on *L. monocytogenes* on both salmon and catfish fillets. In catfish, an average reduction of 1.5 log<sub>10</sub> cfu/g was achieved within 30 minutes after application. In whole catfish a reduction between 1.4 and 2.0 log<sub>10</sub> cfu/g (10 °C) was achieved with a bacteriophage concentration of 2×10<sup>7</sup> pfu/g. On raw salmon fillet tissue treatment with 10<sup>7</sup> pfu/g resulted in a 2 log<sub>10</sub> cfu/g reduction and 10<sup>8</sup> pfu/g resulted in a 3.5 log<sub>10</sub> cfu/g reduction of *L. monocytogenes*. *Listeria* reductions were proportional with the applied bacteriophage concentration. It was demonstrated that P100 was able to reduce *L. monocytogenes* on raw fish fillets as a function of bacteriophage dose, inoculum level, and contact time, while temperature had a minor effect.

#### 7.4. Considerations of studies on efficacy

According to the EFSA guidance (EFSA Panel on Biological Hazards (BIOHAZ), 2010), the processing conditions used to evaluate the efficacy must be comparable with those for which the formulated product is intended and tests must be made with inoculated target microorganisms, taking into account strain diversity. This can be achieved using different strains or cocktails of strains and strains isolated from the surface of the foods to be treated. The studies included in the evaluation examined activity of the formulated product against only two strains of *L. monocytogenes*. Further, strain Scott A is not considered as being representative of recent foodborne outbreaks or of average growth, resistance and survival characteristics for *L. monocytogenes* strains (Barbosa et al., 1994; Lianou et al., 2006), produces aberrant morphological colonies on selective media (Loessner et al., 1988), and spontaneously produces cell wall deficient L-forms (Dell'Era et al., 2009). No *L. monocytogenes* strains of fish origin were included in the studies evaluated.

Soni et al. (2010) concluded that “since there is high diversity of *L. monocytogenes* isolates that may occur in catfish fillets and in processing plants, further work is needed to determine the ability of P100 in eliminating the diverse set of isolates of *L. monocytogenes* occurring in these conditions.” In addition, Soni and Nannapaneni (2010) indicated that “experiments with whole fillets (to mimic the commercial fillet processing operation) are needed to test the efficacy of P100 against the wide range of *L. monocytogenes* isolates that frequently originate in salmon fillet processing facilities.”

The studies, that were considered and evaluated in this Opinion (Soni and Nannapaneni, 2010; Soni et al., 2010), used bacteriophage concentrations of 10<sup>8</sup> pfu/g on raw salmon fillet tissue inoculated with 2-4 log<sub>10</sub> cfu/g *L. monocytogenes* and resulted in reductions of 1.8-3.5-log<sub>10</sub> cfu/g. Reductions on raw catfish fillet tissue treated with 7.3 log<sub>10</sub> pfu/g P100 were 1.4-2.3 log<sub>10</sub> cfu/g when the inoculated level was 4.3 log<sub>10</sub> cfu/g, while the levels of 3 and 5 log<sub>10</sub> pfu/g had either no or only minor effects. The following need to be noted: natural *L. monocytogenes* contamination is usually present on fish at levels lower than those evaluated; natural contamination on fish is not spread evenly or uniformly; the non-motile bacteriophage particles need to come in contact with bacterial cells for action. Thus, the efficacy of the proposed Listex™ P100 treatment may need to be evaluated on fish containing lower levels of *L. monocytogenes* contamination in order to determine efficacy under conditions more representative to real life. This is also important because *L. monocytogenes* is a psychrotroph and survivors may grow during refrigerated storage of fish.

As discussed in Appendix C, main potential sources of fish product contamination include the raw material and the processing environment. However, the initial origin of processing plant contamination remains undetermined (Autio et al., 1999; Autio et al., 2003). In general, *L. monocytogenes* contamination of the processing environment and final product may be variable among processing plants as it may originate from incoming raw materials (e.g. fish, water, ice), other materials (e.g. packaging, additives), biofilms on equipment and other surfaces, and to a lesser extent employees in the production line. Thus, since *L. monocytogenes* contamination of processed fish cannot be attributed to a single source or processing step, no single decontamination treatment could solve the

problem. Further, since contamination can occur at multiple steps, re-contamination of fish post-decontamination may also occur; this might diminish or negate the anti-listerial contribution of previous decontamination treatments. Therefore, any anti-listerial decontamination treatment(s) would need to be verified as useful at the production step implemented as well as for its contribution to reduction of contamination in the finished product and to reduction of listeriosis associated with consumption of fish. Since the length of time that the bacteriophage remains active was not sufficiently documented in the studies examined, the overall efficacy of the Listex™ P100 decontamination could not be assessed throughout the fish processing chain.

Overall, the impact of treating raw fish with Listex™ P100, as early in processing as possible, on the contamination of the final product and its impact on listeriosis reduction needs to be established. Processors also would need specific guidance for proper implementation of the treatment.

## 7.5. Conclusions

- Based on the selection criteria, a total of two of the six submitted papers were included in the assessment of the efficacy of Listex™ P100 as a decontaminating agent against *L. monocytogenes* on raw fish.
- The two laboratory studies, selected for evaluation were classified as of ‘low strength of evidence’. According to the study selection criteria, when a study is classified as of ‘low strength of evidence’, “data are indicative of a disinfectant effect that may be reproducible in practice, but individually do not allow definitive conclusions on risk reduction.”
- The data presented in the two selected studies indicated that the bacteriophage Listex™ P100 was listericidal on inoculated fresh catfish and salmon fillet samples under laboratory conditions.
- *Listeria* reductions achieved increased with applied bacteriophage concentration and were affected by pathogen inoculum level and contact time, while temperature (4 °C and 22 °C) had a minor effect.
  - On raw salmon fillet tissue, a bacteriophage concentration of  $10^8$  pfu/g yielded 1.8-, 2.5-, and 3.5- $\log_{10}$  cfu/g reductions of *L. monocytogenes* from its initial loads of 2, 3, and 4  $\log_{10}$  cfu/g at 4 °C or 22 °C.
  - On raw catfish fillet tissue, bacteriophage P100, applied at 7.3  $\log_{10}$  pfu/g at 4 °C, reduced the inoculated (4.3  $\log_{10}$  cfu/g) *L. monocytogenes* by 1.4 to 2.3  $\log_{10}$  cfu/g; the levels of 3 and 5  $\log_{10}$  pfu/g had either no or only minor effects.
  - Bacteriophage contact time of 30 min caused greater than 1  $\log_{10}$  cfu/g reduction in *L. monocytogenes*, while reductions at 15 min contact time were less than 1  $\log_{10}$  cfu/g of catfish fillet.
- The data provided were not adequate to allow conclusions on the fate of the bacteriophage P100 and of surviving *L. monocytogenes* during product storage after treatment.
  - *Listeria monocytogenes* counts showed an upward trend in treated catfish fillets stored for 10 days at 4 °C or 10 °C, which was not evident in treated salmon fillets stored for 10 days at 4 °C. It is unclear whether these counts were due to total prevention of bacterial growth or to the balance between growth and death of bacterial cells.
  - The bacteriophage P100 titre remained stable on raw salmon fillet samples for 4-7 days and showed slight reductions during days 7-10 at 4 °C. In contrast, P100 titres decreased during the first 4 days of the 10-day storage period of treated catfish samples at 4 °C and 10 °C, and remained stable during days 4-10.

- The two studies considered had the following limitations:
  - Experiments were conducted exclusively under laboratory conditions with the objective of proof of concept.
  - The results were not validated under pilot plant or industrial conditions.
  - These studies selected for assessment evaluated only two types of fresh fish and conditions such as tissue or skin differences or other variables among other types of fresh fish based on composition, muscle properties, etc. were not considered.
  - Replication was limited and sizes of samples were mostly small (5 g and 10 g of fresh, approximately 2 cm<sup>2</sup> top surface area, catfish and salmon, respectively), and only a limited number of larger samples were treated. Such small samples may not allow evaluation of uniformity of application and variability of activity.
  - Although previous studies may have found bacteriophage P100 effective against more strains, only a two-strain, two-serotype mixture of *L. monocytogenes* was tested and no strains of fish origin were included in the studies evaluated. This does not allow consideration of strain variability in terms of survival ability and growth.
- Levels and distribution of natural *L. monocytogenes* contamination on fresh fish may be lower and not as uniformly distributed as were those evaluated in the studies examined. Therefore, the proposed Listex™ P100 levels may not be adequately efficacious in naturally and randomly contaminated fish with lower levels of this psychrotrophic pathogen.
- No evidence was provided to demonstrate the impact of treating raw fish on *L. monocytogenes* contamination levels of finished product.
- The continuous effectiveness of Listex™ P100 against *L. monocytogenes* during repeated use, and the potential for selection and dominance of strains naturally resistant to bacteriophage P100 were not evaluated.
- Overall, the documentation and evidence provided is not adequate to allow estimation of potential human listeriosis risk reduction by treating raw fish with Listex™ P100.

## **8. The potential emergence of reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials linked to the use of the substance**

### **8.1. Evaluation including comments to application**

#### **8.1.1. Treatment of listeriosis**

Because of the relatively few prospective trials on the efficacy of different antibiotic regimens for the treatment of invasive listeriosis, the choice of antibiotic for first-line therapy is still debatable (Mylonakis et al., 1998). It is generally agreed that the most effective antibiotics are ampicillin/amoxicillin or penicillin G in combination with an aminoglycoside, which is classically gentamicin (Charpentier and Courvalin, 1999; Hof, 2003). For patients that cannot be treated with penicillins, second-choice antimicrobials include trimethoprim in combination with a sulphonamide such as sulphamethoxazole (Hof, 2003). Favourable results have also been reported on occasions with tetracyclines and erythromycin (Lorber, 1997). Treatment with fluoroquinolones is controversial. Favourable in vivo effects with moxifloxacin have been observed in experimental rabbits, but conversely the development of listerial meningitis during ciprofloxacin treatment has also been reported (Grumbach et al., 1999).

### 8.1.2. Resistance to biocides and therapeutic antimicrobials

#### 8.1.2.1. Biocides

There is evidence, which indicates that, the use of biocides, and in particular Quaternary Ammonium Compounds (QACs), is linked to the emergence and persistence of strains of *L. monocytogenes* with decreased susceptibility to such compounds. Increased resistance to disinfectants in *L. monocytogenes* within the poultry processing environment was reported by Earnshaw and Lawrence (1998); Lemaître et al. (1998) reported a higher proportion of isolates of *L. monocytogenes* with enhanced resistance to biocides in isolates from poultry and food than from humans; and Mereghetti et al. (2000) reported on the occurrence of isolates of *L. monocytogenes* with decreased sensitivity to QACs in isolates from food. In contrast, in a comprehensive review of the use of biocides in the food processing industry, Holah et al. (2002) reported that there were no differences in susceptibility to biocides in strains of *L. monocytogenes* and *E. coli* isolated from factory environments in the UK over a three-year period when compared to the levels in control laboratory strains of these organisms.

#### 8.1.2.2. Therapeutic antimicrobials

Tetracycline resistance is the most frequent resistance trait in clinical isolates. In a study in the UK of 1 288 clinical isolates made between 1967 and 1990, 33 isolates were found to exhibit resistance to this antimicrobial (MacGowan et al., 1990). In a more recent study in France, 1.27 % of isolates exhibited resistance to tetracyclines, with resistance to both tetracyclines and fluoroquinolones emerging (Morvan et al., 2010). The first strain of *L. monocytogenes* with multiple resistance to therapeutic antimicrobials was reported in 1990. The strain, a clinical isolate originating in France in 1988, exhibited resistance to chloramphenicol, erythromycin, streptomycin and tetracyclines. Resistance was transferable to other strains of *L. monocytogenes*, and also to enterococci and *S. aureus* (Poyart-Salmeron et al., 1990). Subsequently sporadic isolates of *L. monocytogenes* with resistance to a range of therapeutic antimicrobials from cases of human infection have been increasingly reported (for review, see Charpentier and Courvalin (1999)).

In contrast, resistance to therapeutic antibiotics in *L. monocytogenes* from retail foods is rare, and was only observed in less than 1 % of the isolates of *L. monocytogenes* in a study of 1 001 *Listeria* isolates from 67 retail food samples (Walsh et al., 2001). In contrast there was a substantially higher incidence of resistance to tetracyclines, and penicillin G (>6 %) in isolates of *L. innocua*, suggesting species specificity in the acquisition of resistance within the genus. In a similar study of 202 isolates of *L. monocytogenes* from food and the environment from 1996 to 2006 in France, only four strains exhibited resistance, with resistance to erythromycin, tetracyclines and trimethoprim observed (Granier et al., 2011).

Resistance to therapeutic antibiotics can emerge in *L. monocytogenes* by the acquisition of three types of mobile elements – self-transferable plasmids; mobilisable plasmids; and conjugative transposons (Charpentier and Courvalin, 1999). Genes conferring resistance to chloramphenicol, macrolides, lincosamides and streptogramins have been transferred to *Listeria* from *Enterococcus-Streptococcus* by plasmid pIP501, and thence between species of *Listeria* (Perez Diaz et al., 1982). Mobilisation of a non-conjugative resistance plasmid of *Bacillus subtilis* between different species of *Listeria* by plasmid pRYC16, which is widespread in *Listeria* spp. has been reported (Vicente et al., 1988); and transfer of the broad host range conjugative transposon Tn916 mediating resistance to tetracyclines from a *Streptococcus* donor strain to *Listeria* has also been reported (Vicente et al., 1988).

To our knowledge there are as yet no reports on the acquisition of resistance genes to therapeutic antibiotics by *Listeria* spp. by bacteriophage-mediated transduction.

### 8.1.3. *Listeria* bacteriophage P100

*Listeria* bacteriophage P100 is regarded as a virulent, lytic broad-host range *Listeria* phage closely related to the broad-range *Listeria* phage A511, and as such is regarded as being not capable of transduction (Klumpp et al., 2008). Previous EFSA Opinions (EFSA, 2009; EFSA Panel on Biological

Hazards (BIOHAZ), 2009) have given the basis to conclude that P100 is safe because it fulfils the requirements that it is a lytic bacteriophage and it is unable to transduce bacterial DNA. These conclusions have been confirmed in the application dossier by Professor M.J. Loessner<sup>7</sup> (Annex 12 of application dossier).

Although experimental data have not been provided, based on the properties of P100 as listed above, it is concluded that the use of this bacteriophage for the removal of *L. monocytogenes* surface contamination of raw fish under the conditions specified by the applicant is unlikely to result in the potential emergence of reduced susceptibility to biocides and/or resistance to key therapeutic antimicrobials.

#### **8.1.4. Reduced susceptibility of *Listeria* strains**

The possibility for selection of P100-resistant *Listeria* variants as a consequence of the use of Listex™ P100 needs consideration. In the case of bacteriophages, most of bacteriophage-insensitive mutants present altered receptors. The wide host-range of P100 suggests that its receptor may be a common trait of the cell envelope and thus might be important for its biological fitness. In that case, modification of the receptor would result in poorly-competing strains.

### **8.2. Conclusions**

- From the documentation provided, it appears unlikely that the use of Listex™ P100 for the removal of *L. monocytogenes* surface contamination of raw fish, under the conditions specified by the applicant, will result in the potential emergence of reduced susceptibility to biocides and/or resistance to key therapeutic antimicrobials. Nevertheless this conclusion may need verification since the applicant has provided very limited data in support of this statement.
- Naturally occurring P100 *Listeria*-resistant variants might be selected as a consequence of the use of Listex™ P100.

### **9. The risk related to the release of the processing plant effluents, linked to the use of the substance, into the environment**

#### **9.1. Evaluation of risk by the applicant**

The applicant has pointed out that on fresh and processed dairy and meat products more than 10<sup>8</sup> viable bacteriophages per gram are often present (Kennedy and Bitton, 1987). Also bacteriophages are often consumed in food in high numbers (Hagens and Loessner, 2007, 2010), are normal commensals of humans and animals, and are especially abundant in the gastrointestinal tract (Letarov and Kulikov, 2009). Taking these and other information into account the applicant concludes that the release of Listex™ P100 into the environment does not involve any risk. The applicant also states “because bacteriophages can only survive and replicate in the presence of their host bacterium, in this case *Listeria* spp., if this host is not present they will disintegrate within a short amount of time due to adsorption, UV light and other substances present in water or soil”. In Annex 11 of the application dossier five studies are cited (Guenther et al., 2009; Loessner et al., 1996; Loessner et al., 1997; van der Mee-Marquet et al., 1997; Zink and Loessner, 1992) following a literature search (OvidSP, PubMed, Scopus) by the applicant on ‘the involvement of the micro-organism in adverse ecological effects’ with respect to P100 and the closely related bacteriophage A511. Based on this literature search, the applicant has concluded that “bacteriophage P100 is probably not involved in any adverse ecological effects”.

Nevertheless certain bacteriophages used as indicators of faecal contamination (e.g., *Microviridae*, *Siphoviridae* and *Myoviridae*) can persist for up to 90 days in water, and under a range of conditions

<sup>7</sup> M. J. Loessner is Professor at the Institute of Food, Nutrition and Health, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland.



of heat and UV (Lee and Sobsey, 2011). Tests to monitor the survival of P100 in processing wastewater and the environment are therefore recommended.

## 9.2. Conclusion

- Scrutiny of the papers cited by the applicant did not reveal any information relating directly to the survival of P100 in the environment following its use for *L. monocytogenes* control, nor was any information provided about its ability to survive in wastewater. As far as can be seen from the application dossier, there have been no studies undertaken by the applicant to assess survival times of P100 at the concentrations proposed for use in processing plant effluents.

## CONCLUSIONS AND RECOMMENDATIONS

### CONCLUSIONS

#### TOR 1: Conclusions in relation to the toxicological safety of the substance

- The Listex™ P100 preparation should not present toxicological problems if used in fish decontamination because:
  - The bacteriophage P100 used as active principle is not regarded as harmful to consumers.
  - The Listex™ P100 fabrication parameters do not include any components or steps that might compromise safe use of the preparation.

#### TOR 2: Conclusions in relation to the efficacy, i.e. does the use of the substance significantly reduce the level of contamination of *Listeria monocytogenes*

- Although the data of the two low strength of evidence relevant studies considered are indicative of a disinfectant effect that may be reproducible in practice, as conducted, the studies do not allow definitive conclusions on the efficacy of the product in reducing *Listeria monocytogenes* counts on raw fish.
- Data presented demonstrated that Listex™ P100 is listericidal (reductions were in the range of 1.4 to 3.5 log<sub>10</sub> cfu/g) on the two inoculated raw fish types (i.e., fresh catfish and salmon fillet samples) tested.
- Among other limitations, the studies evaluated only a two-strain, two-serotype mixture of *L. monocytogenes* (no strains of fish origin were included).
- The data provided were not adequate to draw firm conclusions on persistence or activity of the bacteriophage P100 during storage of treated fish samples.
- The data provided were not adequate to draw firm conclusions on the fate of surviving *L. monocytogenes* organisms during storage of treated fish samples.
- Efficacy of the proposed Listex™ P100 treatment against levels of *L. monocytogenes* usually expected to occur naturally on fish was not studied.
- No evidence was provided to demonstrate the impact of treating raw fish on *L. monocytogenes* contamination levels in the finished product.
- Overall, the evidence provided is not adequate to allow estimation of potential human listeriosis risk reduction by treating raw fish with Listex™ P100.

**TOR 3: Conclusions in relation to the potential emergence of reduced susceptibility to biocides and/or resistance to therapeutic antimicrobials linked to the use of the substance**

- Although experimental evidence on this issue is very limited, the proposed use of Listex™ P100 is considered unlikely to result in the emergence of reduced susceptibility to biocides and/or resistance to key therapeutic antimicrobials. Nevertheless this conclusion may need verification.
- Naturally occurring P100 *Listeria*-resistant variants might be selected as a consequence of the use of Listex™ P100.

**TOR 4: Conclusions in relation to the risk related to the release of the processing plant effluents, linked to the use of the substance, into the environment**

- No information was provided on the survival of P100 in processing wastewater or the environment.

**RECOMMENDATIONS**

- Considering the limitations of the proof of concept investigations evaluated by the Panel, pilot and industrial scale studies should be considered to address parameters such as types and size of fish, stage of processing for application of Listex™ P100, multiple and more appropriate strains used for inoculation, contamination levels comparable to those naturally present, fish sample size and repetitions.
- Verification is needed that treatment of raw fish with Listex™ P100 will have an impact on *L. monocytogenes* contamination levels in the final fish product.
- The persistence or activity of P100 as well as potential changes in *L. monocytogenes* counts should be further evaluated during storage of treated fish samples.
- The continuous effectiveness of Listex™ P100 against *L. monocytogenes* and the potential for selection and dominance of strains naturally-resistant to P100 should be monitored.
- This additional knowledge should be provided in order to understand the influence of variables associated with practical applications, and, thus, allow commercial operators to select conditions of use that are validated and verified according to HACCP principles.

**DOCUMENTATION PROVIDED TO EFSA**

1. Letter Ref. Ares(2011)889537 received on 19 August 2011 including the request from the Commission and the application dossier from Micros BV (the Netherlands) “Submission of data for the evaluation of the safety and efficacy of Listex™ P100 for the removal of *Listeria monocytogenes* surface contamination on raw fish”.
2. Reply to questions posed on 17 October 2011 by the Working group to the Contact Person at Micros BV (the Netherlands). Received from Micros on 2 November 2011.
3. Non-confidential information related to the production process of Listex™ P100. Received from Micros BV on 18 November 2011.
4. Confidential information related to the raw materials used in the production process of Listex™ P100. Received from Micros BV on 1 March 2012.

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## APPENDICES

### A. PUBLIC HEALTH CONCERNS ASSOCIATED WITH LISTERIOSIS AND SEAFOOD

*Listeria monocytogenes* is the causative agent of human listeriosis, a potentially fatal infection (Farber and Peterkin, 1991). The disease is predominantly transmitted through consumption of contaminated foods, and is one of the major causes of death from a preventable foodborne illness with case-fatality rates that may exceed 30 %, especially amongst vulnerable groups. Over the past decade an increase in reported cases of human listeriosis has been documented (Denny and McLauchlin, 2008).

A total of 1 601 confirmed human cases of listeriosis were reported in 2010 (EFSA and ECDC, 2012). This represented a 3.2 % decrease compared to 2009. The overall EU notification rate was 0.35 cases per 100 000 population. The overall EU notification rate of confirmed cases of listeriosis varied from 0.33 to 0.38 cases per 100 000 population between 2006-2010 for countries reporting data for five consecutive years. The notification rate was highest in those aged over 65 years, covering 60.2 % of all reported cases, while 6.7 % of cases were detected in the age group 0-4 years with the majority of the cases (96.3 %, n=108) in this latter group being infants of age <1 year. The highest case fatality was reported for the age groups 0-5 years (22.9 %) followed by 45-64 years (19.1 %) and 65 plus years old (17.3 %). In total, 98 % of confirmed *L. monocytogenes* cases with known importation status were of domestic origin. The transmission route was stated for 132 (8.26 %) confirmed cases. Of those, 87 cases were infected with *L. monocytogenes* via suspected food. Of these cases, cheese was mentioned as the suspected vehicle for 13 cases, milk and fish for one case each, while for the remaining cases no information on the food source was provided.

The BIOHAZ Panel concluded that worldwide, infection has been reported through consumption of contaminated food and a wide range of food types have been implicated including processed fish and shellfish (Brett et al., 1998; Ericsson et al., 1997; Facinelli et al., 1989; Farber et al., 2000; Lyytikäinen et al., 2006; Misrachi, 1991; Mitchell, 1991; Riedo et al., 1994; Tham et al., 2000). Fish and shellfish implicated in disease transmission included processed, ready-to-eat (RTE), able to support growth of this bacterium, and likely to have been contaminated at the point of processing.

*Listeria monocytogenes* is widespread in the environment and commonly occurs in surface waters (Colburn et al., 1990; Wilkes et al., 2011), which will consequently contaminate estuarine and coastal waters (Beleneva, 2011; Bou-m'handi et al., 2007; Colburn et al., 1990; El-Shenawy, 2006; El Marrakchi et al., 2005; Hansen et al., 2006; Rodas-Suarez et al., 2006; Rorvik et al., 1995). Survival in seawater has been reported for a few days, and is dependent on temperature, salinity and UV light exposure (Bremer et al., 1998; Hansen et al., 2006; Hsu et al., 2005).

As summarized by the BIOHAZ Panel, raw fish and smoked fish have been reported as contaminated by *L. monocytogenes* at varying rates (0-45 %), examples of which are shown in Table 1.

Literature data on *L. monocytogenes* in raw, unprocessed fish report prevalence of the pathogen, but information on concentration is lacking; concentration data are primarily available for RTE fishery products. Although the prevalence of *L. monocytogenes* in cold-smoked fish is often high, concentrations of microorganisms are typically low (USDA, 2012). For instance, Eklund et al. (1995) found that although as many as 48 out of 61 samples of USA cold-smoked salmon contained *L. monocytogenes*, its concentration ranged from 0.3 to 34.3 colony forming units (cfu)/g with a mean of 6.2 cfu/g. Similarly low levels were found in Danish cold-smoked salmon, where 34 of 64 samples were positive, with 28 of them containing fewer than 10 cfu/g, 5 samples containing between 10 and 100 cfu/g, and one sample containing between 100 and 1 000 cfu/g (Jorgensen and Huss, 1998). Fewer than 10 cfu/per gram were found in smoked finfish in USA (Jinneman et al., 1999). Furthermore, Johansson et al. (1999) reported that 50 % of vacuum-packed smoked fish contained <100 cfu/g. In a survey of smoked fish (not specified hot- or cold-smoked) on the German market, 27 samples (of 380) were positive for *L. monocytogenes*, with five samples containing less than 1 cfu/g, 14 samples containing between 1 and 100 cfu/g, and four samples containing between 100 and 10<sup>4</sup> cfu/g; in four samples, levels exceeding 10<sup>4</sup> cfu/g were found (Notermans et al., 1998; Teufel and Bendzulla, 1993).



Gombas et al. (2003) surveyed eight categories of RTE foods from retail markets in two states of the USA over a 14-23 month period for presence of *L. monocytogenes*. Total numbers of positive seafood salads and smoked seafood samples were 229 out of 5 090 tested, compared to 348 out of 26 615 tested for all other six foods combined. Most samples of all foods had concentrations of the pathogen of less than 10 cfu/g. Counts of 10-10<sup>6</sup> cfu/g were detected in 17 smoked seafood samples and 24 samples of seven other products.

EU legislation (Regulation (EC) No 2073/2005<sup>8</sup>) lays down food safety criteria for *L. monocytogenes* in RTE foods, as follows:

- in RTE products intended for consumption by infants and for special medical purposes *L. monocytogenes* must not be present in 25 g;
- *Listeria monocytogenes* must not be present at levels above 100 cfu/g during the shelf life of other RTE products;
- for RTE foods that support the growth of the bacterium, *L. monocytogenes* may not be present in 25 g at the time of leaving the production plant. However, if the producer can demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout shelf life this criterion does not apply; and
- for RTE foods that support growth of *L. monocytogenes*, the microbiological criterion to be applied depends on the stage in the food chain and whether the producer has demonstrated that *L. monocytogenes* will not multiply to levels of 100 cfu/g, or above, during shelf life.

As summarized by the BIOHAZ Panel from the EU Summary Report (EFSA and ECDC, 2012), in 2010, 11 MSs reported data on findings of *L. monocytogenes* in RTE fish products. The products tested were mainly smoked fish, except for Romania (cooked fish). At processing level, the proportion of single samples of fishery products in non-compliance with the criteria (absence in 25 g) has increased consistently from approximately 4 % in 2007 to 9.6 % in 2010. Both in single samples (9.6 %) and for batch-based sampling (4.5 %), the highest level of non-compliance was observed in RTE fishery products when compared to the other food categories. The same observations can be made at retail level. The highest levels of non-compliance with the criterion of ≤100 cfu/g among single samples, were also observed in RTE fishery products (1 %) in 2010. For the batch-based sampling at retail the highest non-compliance was reported for soft and semi-soft cheeses (0.8 %) followed by RTE products of meat origin other than fermented sausage (0.6 %) as well as other RTE products (0.2 %).

The presence of *L. monocytogenes* in fish was detected in eight out of 14 qualitative investigations. A total of 2 938 samples were tested by detection method. The Netherlands submitted near a third of these investigations (1 001 samples) from various types of fish (trout, mackerel, salmon, herring and eel) with a prevalence of 6.1 % compared to 7.0 % in 2009. High proportions of *L. monocytogenes* positive samples were reported at the processing plant by Ireland (28.3 %) and Denmark (22.2 %).

Seven out of eleven quantitative investigations reported levels of *L. monocytogenes* above 100 cfu/g. Overall, 1.3 % of 2 607 samples tested quantitatively were found to exceed the limit of 100 cfu/g, compared with 0.6 % in 2009 and 0.5 % in 2008. The proportion of samples containing the bacteria at concentrations above the limit of 100 cfu/g ranged from 0.1 % to 18.8 %, with the highest level in samples of smoked fish at retail in Denmark.

Five MSs reported investigations in other fishery products. *L. monocytogenes* was detected in 5.7 % of the 1 092 samples taken under qualitative investigations. Estonia reported the highest level of positive

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<sup>8</sup> Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. OJ, L 338, 22.12.2005, p. 1-29.

findings with 18.9 % positive samples taken at processing plant. Three of the five MSs also found *L. monocytogenes* at levels above 100 cfu/g with incidence ranging between 0.6 % and 7.1 %.

*Listeria monocytogenes* was detected by qualitative testing in crustaceans, molluscan shellfish, and in other fishery products. Germany reported *L. monocytogenes* in 2.0 % and 3.1 % of crustaceans at retail and processing plant level, respectively, and Hungary reported 1.2 % of molluscan shellfish tested positive at retail.

In 2010 and 2011, an EU-wide baseline survey on *L. monocytogenes* in RTE foods has been carried out targeting smoked and gravad fish, soft and semi-soft cheeses, and heat-treated meat products that have been handled between the heat treatment and packaging. The results of this survey will provide further valuable information on the occurrence of *L. monocytogenes* in these RTE food categories perceived as being at high risk regarding *Listeria* contamination.

**Table 1:** Examples of rates of contamination of raw and smoked fish with *L. monocytogenes*

Food type	Stage in food chain	Nb tested	Nb (%) <i>L. monocytogenes</i> detected	Reference
<b>RAW FISH (country of origin)</b>				
Raw unfrozen aquacultured catfish, salmon, tilapia and trout (USA)	Ready to cook, local and internet vendors	272	34 (13 %)	Pao et al. (2008)
Raw fish post freezing (Germany)	Ready to eat, retail (sushi)	250	3 (1 %)	Atanassova et al. (2008)
Raw catfish fillets (USA)	Ready to cook, factory	80	36 (45 %)	Chen et al. (2010)
Raw fish (Turkey)	Ready to cook, retail market	78	14 (18 %)	Yucel and Balci (2010)
Raw fish (Japan)	Ready to eat, retail (sushi)	701	38 (5 %)	Miya et al. (2010)
Raw fish (Denmark)	Ready to smoke, factory	21	5 (24 %)	Wulff et al. (2006)
Raw fish (USA)	Ready to smoke, factory	102	9 (9 %)	Norton et al. (2001)
Raw salmon (Denmark)	Ready to smoke, factory	30	0	Fonnesbech Vogel et al. (2001a)
Whole raw fish (Mexico)	Sea caught	66	3 (5 %)	Rodas-Suarez et al. (2006)
Whole farmed rainbow trout (Finland)	Farm caught	510	15 (15 %)	Miettinen and Wirtanen (2006)
Raw and dried fish (Iceland)	Ready to eat, retain and from factories	55	6 (11 %)	Hartemink and Georgsson (1991)
Raw fish (Japan)	Ready to smoke, factory	94	0	Nakamura et al. (2006)
Raw catfish fillets (USA)	Ready to cook, factory	240	90 (37 %)	Chou et al. (2006)
<b>SMOKED FISH (country of origin)</b>				
Cold smoked fish (USA)	Finished product	96	11 (12 %)	Norton et al. (2001)
Cold smoked salmon (Norway and Faroe Islands)	Finished product	88	30 (34 %)	Fonnesbech Vogel et al. (2001a)
Cold smoked fish (Japan)	Finished product	59	4 (7 %)	Nakamura et al. (2006)
Cold smoked salmon and trout (Denmark and Faroe Islands)	Finished product	74	13 (18 %)	Wulff et al. (2006)
Cold smoked fish (USA)	Finished product	300	28 (9 %)	Hu et al. (2006)
Cold smoked fish (Canada, Norway, Philippines and UK)	Finished product	240	51 (21 %)	Heinitz and Johnson (1998)
Hot smoked fish (Canada, Norway, Philippines and UK)	Finished product	215	19 (9 %)	Heinitz and Johnson (1998)

## B. BIOLOGY OF BACTERIOPHAGES AND THEIR USE AS ANTIBACTERIAL AGENTS

### a. Biology of bacteriophages

Bacteriophages are viruses attacking bacteria and, as such, they are intracellular obligate parasites. Their infective form (**the virion**) is composed of a nucleic acid (usually double stranded DNA) surrounded by a proteinaceous coat (**the capsid**). Most bacteriophages possess a tail to allow injection of the nucleic acid into the cytoplasm, which will constitute the intracellular and metabolically active viral form.

Bacteriophages are present wherever bacteria live and thus they are ubiquitous in the environment and in body cavities (digestive tract and vagina) where they thrive on the indigenous microbiota. Furthermore, they are routinely consumed with fermented foods where they develop at the expense of the fermentation starter bacteria; in fact, they constitute the most important single cause of failures in dairy fermentations. They have even been used for human therapy in certain eastern countries, although not currently licensed in EU and USA (Brussow, 2005). Limited controlled clinical studies are available although no serious side effects have been ascribed. The few minor side effects reported (Cislo et al., 1987; Slopek et al., 1987) for therapeutic phages may have been due to the liberation of endotoxins from Gram-negative bacteria lysed in vivo by the phages. Temperate bacteriophages, however, may enhance the virulence of some bacterial pathogens as is explained below.

In general, virions are able to remain in the environment for long periods of time due to their lack of metabolism. However, most are dependent on the presence of divalent cations and susceptible to temperature shifts and proteases, which are frequently produced by environmental microorganisms. In any case, inactivated bacteriophage particles eventually break down into amino acids and nucleosides and thus they do not represent a risk for the environment (Carlton et al., 2005).

The encounter of a bacteriophage with its host is a random event and resulting infection relies on specific recognition. This implies that bacteriophages have narrow host ranges, rarely expanding further than the species or genus level for Gram-positive and Gram-negative bacteria, respectively.

Upon infection, bacteriophages may follow a **lytic or a lysogenic cycle**; those that can only follow the lytic cycle are known as **virulent** while those that may choose lysogeny are called **temperate** bacteriophages. The lytic cycle ends with breakage of the host bacterial cell wall and liberation of the phage progeny, which may fluctuate between a few tens to a few hundred virions. Killing of the host may also be accomplished without phage development as a consequence of massive viral adsorption, which will harm the cell wall and membrane and produce loss of the membrane electric potential; this process is called **lysis from without** to distinguish it from the usual **lysis from within**.

Temperate bacteriophages synthesize a repressor protein that silences most bacteriophage genes and results in abortion of the lytic cycle and in immunity to superinfection by related bacteriophages. This is usually followed by integration of the phage DNA into the host genome, which ensures transmission to the host-offspring.

Some temperate bacteriophages harbour other genes that are also expressed during lysogeny. These may confer new properties to their hosts (**lysogenic conversion**), this being especially relevant for those that encode virulence factors, such as the diphtheria toxin encoded by the bacteriophage  $\beta$  of *Corynebacterium diphtheriae*, the verocytotoxin-producing *Escherichia coli* bacteriophages, and many others.

Bacteriophage involvement in host-acquisition of new properties also occurs through **transduction**, where bacterial DNA is transferred from cell to cell inside viral capsids. This process is extremely rare for bacteriophages that package unit genomes, i.e. those with cohesive

ends and with terminally redundant nonpermuted genomes, due to very tight recognition of the DNA extremes, while it is more frequent in bacteriophages that have circularly permuted genomes, because in this case the capsid admits as much DNA as can be accommodated, which is more than the unit genome and, by definition, has to have a more relaxed recognition of the DNA ends.

Bacteriophage attack results in selection of bacteriophage insensitive mutants (BIMs), which usually have altered receptors in their cell walls. Devoted bacteriophage resistance systems have also been described, especially in bacteria subjected to frequent challenges, such as fermentation starter cultures. Among them are restriction-modification (R-M) and abortive infection (Abi), both of which may be plasmid encoded, thus facilitating their spread. Furthermore, the so-called CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) sequences keep a memory of past infections in the form of short DNA sequences from the corresponding bacteriophages. Expression of these is believed to produce antisense RNA segments that, upon pairing with the mRNAs of the incoming bacteriophages, would block transcription and thus, infection.

#### **b. Bacteriophage contamination of animal derived products**

The presence of bacteriophages in meat is a reflection of the bacteria that contaminate the food-animals previously and after slaughter. They appear to be especially abundant on poultry surfaces because the skin is retained on the carcasses and in ground beef due to the processing and breakage of the natural barriers to contamination (Hsu et al., 2002; Kennedy and Bitton, 1987). The bacteriophages found are mainly those infecting enteric bacteria, especially *E. coli*, *Salmonella* and *Campylobacter*. The frequency of isolation consistently exceeds 50 % of the samples, while the concentrations vary from  $10^1$  to  $10^6$  bacteriophages/cm<sup>2</sup> of skin, but are mainly in the  $10^2$  range (Hsu et al., 2002; Tsuei et al., 2007). It appears that freezing drastically reduces bacteriophage recovery (Atterbury et al., 2003).

Bacteriophages specific for environmental bacteria such as *Staphylococcus aureus* and psychrotrophic microorganisms such as *Pseudomonas* spp. may be present as well, especially on meats stored at low temperature (Greer, 1983; Kennedy and Bitton, 1987).

Isolation of bacteriophages from seafood has been reported as well, although data on this topic are scarcer than for meat. For example, enteric bacteriophages were recovered from water-filtering molluscs such as mussels and oysters (Crocì et al., 2000), while *Pseudomonas* bacteriophages were isolated from fish meat (Delisle and Levin, 1969).

Finally, bacteriophages targeting the fermentation starter bacteria are abundant in dairy products. Their main source appears to be the milk or the bacteriophages that are endemic in the factory rather than the starter inocula (Madera et al., 2003).

#### **c. Bacteriophages as antibacterial agents in foods**

Bacteriophages used in food-decontamination have to possess at least the following properties: (i) be virulent to avoid lysogenization leading to survival and lysogenic conversion of the infected host; (ii) have unit genome packaging to minimize transduction; (iii) cover a wide range of susceptible bacteria, which in practical terms means many strains within a single species or, at most, several species of a genus; (iv) generate numerous progeny; and, (v) have a short lytic cycle to allow multiple rounds of infection-host lysis (EFSA Panel on Biological Hazards (BIOHAZ), 2009).

On the other hand, bacteriophages are obligate parasites and thus their development depends on the growth conditions of their hosts. The problem may then arise when the bacteria are in the stationary phase or growing very slowly, which may be the predominant conditions in foods due to the preservation procedures: low temperatures, low water activity whether caused by salt or other means, etc. It is, however, believed that, in the environment, bacteria are in a state similar to stationary phase most of the time and this does not preclude high bacteriophage abundance in

natural ecosystems. In fact, bacteriophage replication, albeit at a reduced rate, has been observed on stationary phase *E. coli* and *P. aeruginosa* cells (Schrader et al., 1997).

Another consideration to bear in mind is the relative host-bacteriophage concentration and the possibility for the latter to diffuse on the surface of solid food during the treatment. Concerning this, two approaches for bacteriophage usage are possible (EFSA, 2009):

**Passive treatment.** Bacteriophages are added in sufficient quantity to cover the whole surface and overwhelm all target bacteria by primary infection and, ideally, by lysis from without. This is the technique to use in case of low-level bacterial contamination and on relatively dry food surfaces that will not allow significant diffusion of progeny bacteriophages. Two advantages of lysis from without are that no bacteriophage development would be necessary for cell killing and that the cytoplasm bacteriophage resistance mechanisms (R-M, Abi, CRISPR) would not be an issue.

**Active treatment.** Elimination of bacteria will rely on successive rounds of development-lysis. Success will depend on a relatively high host concentration and the possibility for the bacteriophage to spread on the material, which will be very much influenced by the moisture and the presence of inert cells on which progeny bacteriophages might become adsorbed. The problem of this procedure may be to define what is ‘a relatively high host concentration’. There are several mathematical models aimed at defining a threshold for efficacy, but their conclusions differ by several orders of magnitude (Kasman et al., 2002; Obeso et al., 2010; Payne and Jansen, 2001; Wiggins and Alexander, 1985). This might be due to the combinations of bacteriophage-host used, to the characteristics of the foods treated or to the assumptions made for modelling. In practice, the treatment efficacy parameters have to be determined experimentally on a case-by-case basis.

Control of undesirable bacteria by bacteriophages has been assayed in most types of foods of animal origin, poultry being the most widely tested. In this case, most studies addressed *Salmonella* and *Campylobacter*, the main concerns in these meats (Goode et al., 2003; Higgins et al., 2005), while those performed on beef and pork products challenged mostly *E. coli* O157, *L. monocytogenes* and the psychrotrophe *Brochothrix thermosphacta* (Abuladze et al., 2008; Anany et al., 2011; Zhang et al., 2010). Work on sea-food involved *L. monocytogenes* almost exclusively (Guenther et al., 2009; Soni and Nannapaneni, 2010; Soni et al., 2010) and those on dairy products focused on *L. monocytogenes* and staphylococci (Carlton et al., 2005; Garcia et al., 2007; Schellekens et al., 2007). The overall conclusion might be that frequently the bacterial titres are reduced significantly and even go below detectable levels, especially when concentrated bacteriophage preparations are used and when the treatment is combined with nisin or proceeds at room temperature, instead of under the usual refrigerated conditions (Bigwood et al., 2008; Dykes and Moorhead, 2002; Garcia et al., 2010). In some cases, bacterial recovery was detected upon prolonged incubation. These survivors were usually not bacteriophage resistant. This suggests that bacteriophages may become adsorbed to the food matrix soon after application, although this does not necessarily mean inactivation, because they can be washed from it and still produce plaques of lysis (Guenther et al., 2009). This behaviour variability impedes the definition of bacteriophages as processing aids or as additives, when applied to foods for decontamination (EFSA, 2009).

Finally, in the 2009 EFSA update on the maintenance of the list of Qualified Presumption of Safety (QPS) microorganisms intentionally added to food or feed (EFSA Panel on Biological Hazards (BIOHAZ), 2009) is concluded that “bacteriophages cannot be included on the QPS list” but rather have to be allocated to a case by case analysis before authorization to be used on foods is granted and this for the following reasons: i) impossibility to place them to precise taxonomical units (genera and species), and ii) impossibility to know a priori whether a particular bacteriophage is strictly virulent, does not harbor potentially harming genes and does not have the possibility to transduce bacterial DNA between hosts.



### C. FISH PROCESSING LINES AND *LISTERIA MONOCYTOGENES* CONTAMINATION

The following material is summarized by the BIOHAZ Panel. *L. monocytogenes* can be present in both fresh and marine water, with the highest prevalence in coastal areas and in polluted waters (Ben Embarek, 1994). In fish, *L. monocytogenes* is commonly found on surfaces of skin, gills, heads and slime, and the contamination of fish most likely depends on the presence of the bacteria in the surrounding waters. Hence, it was suggested that raw fish is the primary source of contamination for RTE fish (Eklund et al., 1995), but this was concluded without molecular tracing of the isolates.

In contrast, a number of other studies using molecular typing techniques have suggested that raw fish is not the major source of final product contamination; rather, contamination occurs primarily during processing (Fonnesbech Vogel et al., 2001a; Fonnesbech Vogel et al., 2001b; Norton et al., 2001). Fish processing is very diverse and includes a number of steps that vary with types of fish and types of intended product. For illustration purposes, examples of generic flow diagrams of processing of live fish, cooled/frozen fish and cold-smoked fish are summarised in Figures 1, 2 and 3, demonstrating the complexity of these processes. It is not possible to outline in detail all possible types of production practices and their variations that are used for different fish products in different countries, because they are numerous and complex as they are both product- and producer-specific. As concluded by the BIOHAZ Panel, the complexity of the fish processing procedures makes possible repeated contamination of the product with *L. monocytogenes* via various routes, which makes control of the pathogen complex and requiring application of a range of control measures at different points in the process (e.g. raw materials-, environment-, staff-, equipment- and product-focused).

*Listeria monocytogenes* strains that contaminate fish are often recovered from processing equipment such as mechanical saws, brining, slicing, dicing, freezing, and packaging machines and conveyors. These machines are therefore considered essential niches of the bacteria and important sites of product contamination (Aguado et al., 2001; Autio et al., 1999; Autio et al., 2003; Lunden et al., 2003a; Lunden et al., 2003b; Miettinen et al., 1999; Norton et al., 2001; Ojeniyi et al., 2000; Rorvik, 2000; Rorvik et al., 1995). Such equipment often has narrow openings and dead-end areas making its dismantling for efficient cleaning and sanitation difficult. In cold smoked fish processing plants, the most contaminated sites are the brining and post-brining areas, and during production the brine solutions become contaminated with *L. monocytogenes* originating from brining machines (Autio et al., 1999; Autio et al., 2003).

Airborne bacterial contamination is also possible in the food processing environment in general (Bjorkroth and Korkeala, 1997; Rahkio and Korkeala, 1997), but published research does not indicate air-mediated contamination as of major importance in *L. monocytogenes* contamination of processed fish.

Furthermore, the employees may play some role in the spreading of *L. monocytogenes* contamination, but the extent of their contribution to processed fish contamination still remains unclear. The gloves of fish handlers were found contaminated, but most likely become contaminated when the workers handle the fish, rather than the gloves themselves being the source of contamination (Autio et al., 1999; Autio et al., 2003).

A letter by Professor L. Gram<sup>9</sup> included in the application dossier (reference letter d.d. 27 Oct 2010), states that, according to their studies in the Danish smoked fish industry, contamination of the product (smoked salmon) predominantly takes place during processing (slicing), after salting and smoking. The raw fish does sporadically harbour *L. monocytogenes* and the raw fish processing area often has a relatively high occurrence of *L. monocytogenes*, but the molecular subtypes on raw fish are in most processing plants different from the subtypes encountered

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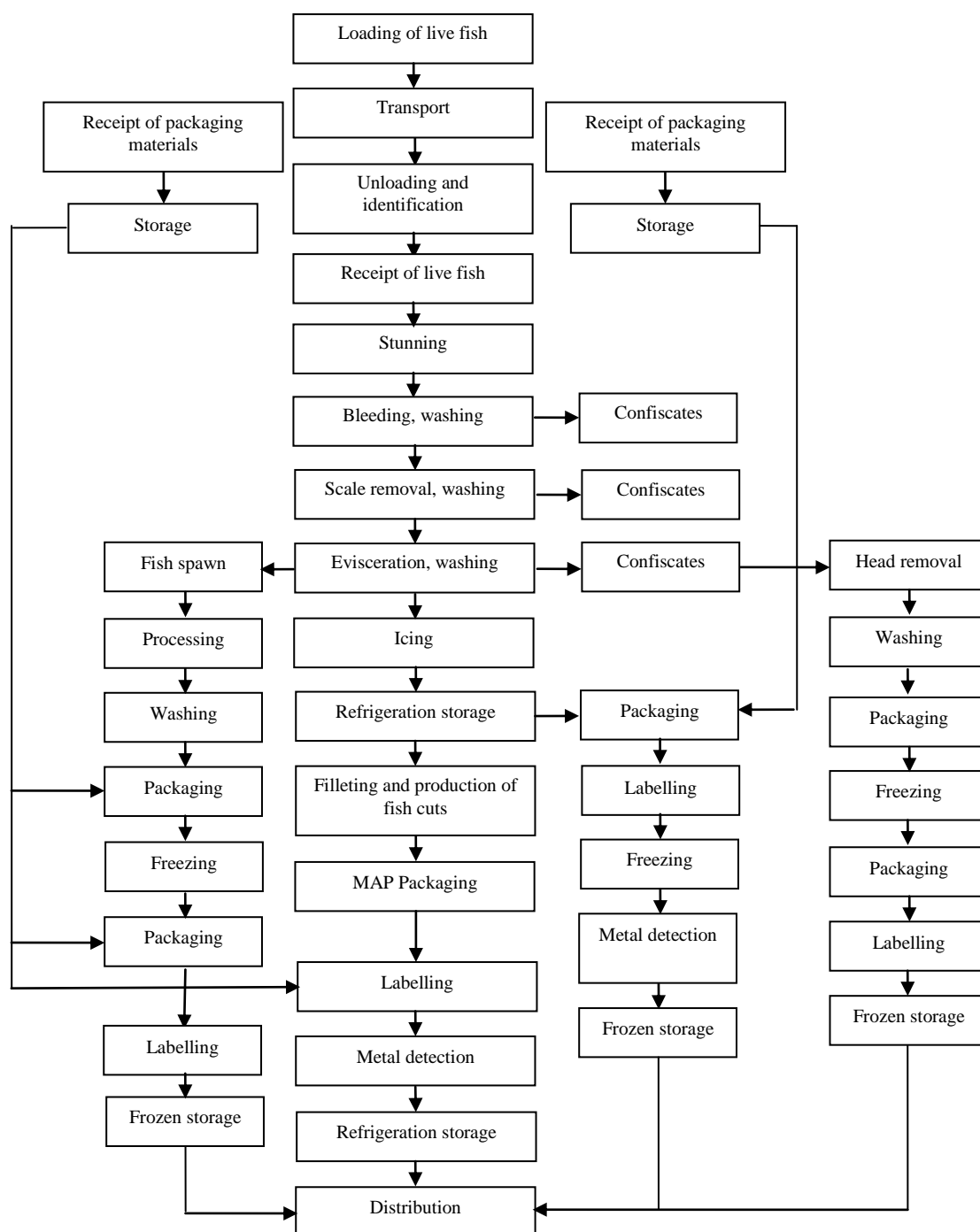
<sup>9</sup> L. Gram is Professor at the Division of Industrial Food Research, Technical University of Denmark (DTU-FOOD), Kgs. Lynby, Denmark.

during processing and on the final product. The original contamination sources of *L. monocytogenes* are not known but it likely enters from almost any outside source (raw fish, trucks, boots, boxes). Reducing this introduction could potentially limit the contamination of the final product. It is further stated in the letter that: procedures (preservation) that prevent growth in the product are essential for safety improvement; processes that eliminate the organism at the end of production would be of interest; and irrespective of such measures, it is of great importance that continued efforts are made to reduce contamination level and prevalence at all steps in production.

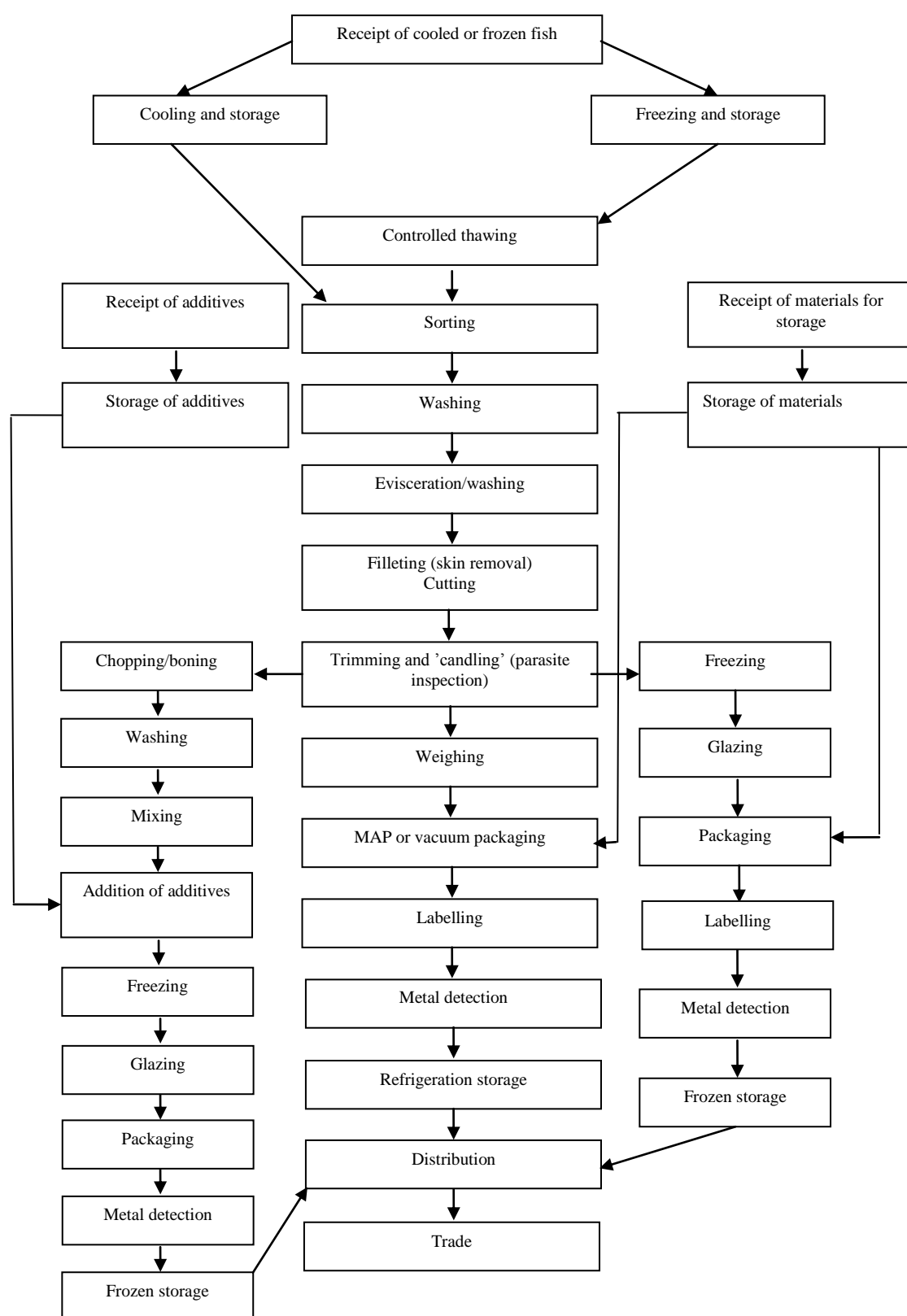
Overall, even though fish product contamination appears to be clearly associated with processing, the initial origin of processing plant contamination remains undetermined (Autio et al., 1999; Autio et al., 2003). *L. monocytogenes* may be introduced into the processing environment and subsequently onto the product through a number of routes, including via incoming raw materials (e.g. fish, water, ice), other materials (e.g. packaging, additives), biofilms on equipment and other surfaces, and to a lesser extent employees handling the product along the production line. Contributing sources may also vary between plants. This is indirectly shown in some published studies. For example, the overall incidence of *L. monocytogenes* in fish processing plants varied from 5.9 % to 22.1 %; roughly >3-fold (Gudbjornsdottir et al., 2004). Also, a Danish study indicated great plant-to-plant variation in contamination rate, thus from some plants all product samples were positive whereas other plants produced products where *L. monocytogenes* was not detected (Jorgensen and Huss, 1998). A similar conclusion was reached by Norton et al. (2001) who visited five United States smoke houses over a 6-month period. Using ribotyping, the authors found that each smoke house harbored its own specific ribotype(s) of *L. monocytogenes*.

It seems that *L. monocytogenes* contamination of processed fish cannot be attributed to a single production step; in such a case a single decontamination treatment could have been applied in order to solve the problem. Rather, because the contamination can occur at multiple steps, re-contamination of the fish post-decontamination during the production process may also occur, which would diminish or negate the anti-listerial effects achieved with the single decontamination treatment previously applied. Hence, any anti-listerial decontamination treatment(s) would need to be specified and justified in terms of the production-processing step or steps where its application(s) is proposed. Since the length of time that the bacteriophage remains active and the levels of *L. monocytogenes* contamination to be inactivated were not sufficiently documented, the overall efficacy of the Listex™ P100 decontamination cannot be assessed throughout the food chain. Thus, the impact of treating raw fish with P100 as early in processing as possible on the contamination of the final product needs to be established. Processors would need specific guidance for proper implementation of the treatment.

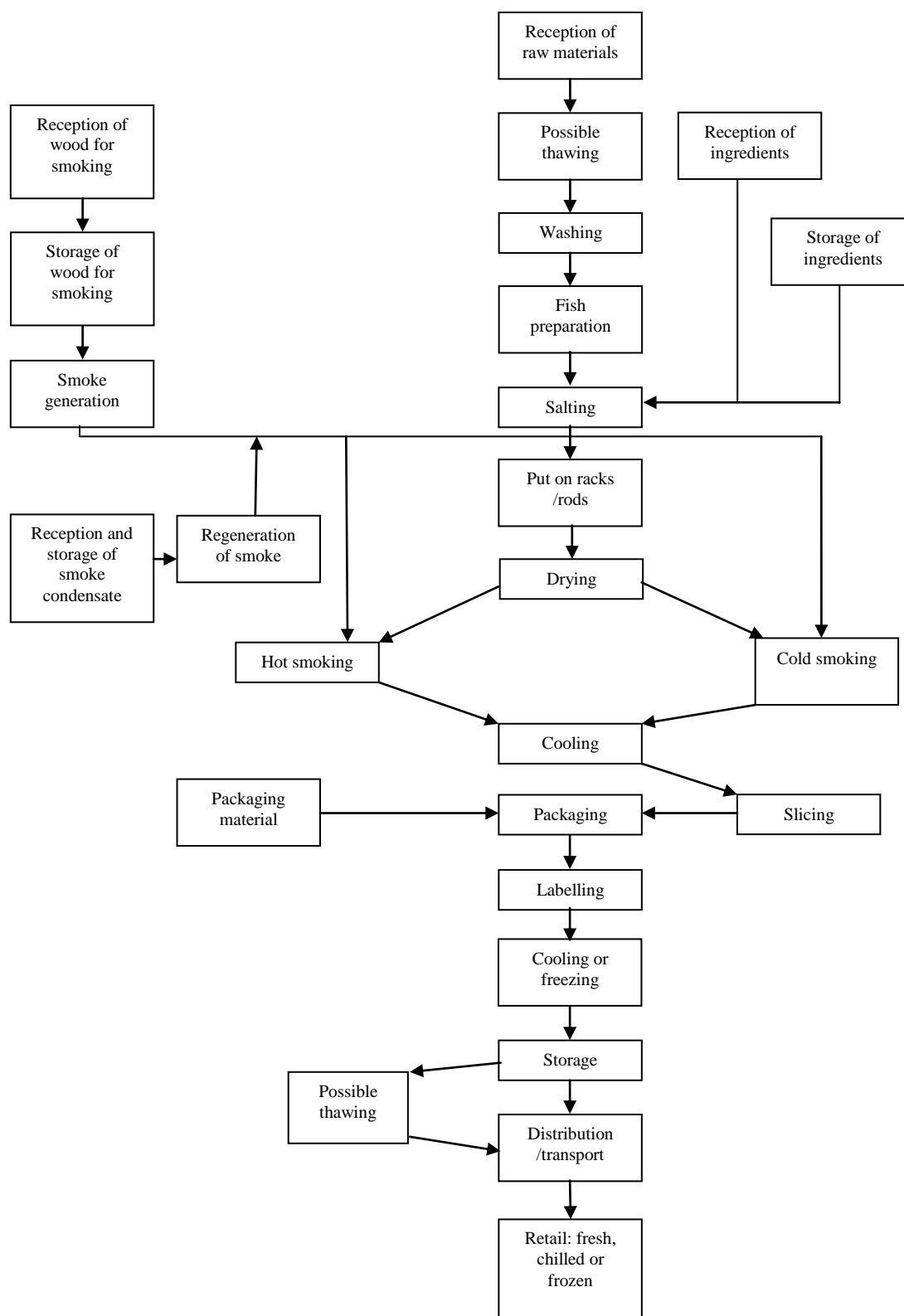




**Figure 1:** Flow diagram of the production of cooled or frozen fish from live fish (Adapted from CAC (2005))



**Figure 2:** Flow diagram of fish fillet and minced fish (mechanically recovered fish flesh) production (Adapted from CAC (2005))



**Figure 3:** Flow diagram of hot smoking, cold smoking and by regenerated smoke preparation lines, including possible slicing operation in the cold smoking line (Adapted from CAC (2005))

## GLOSSARY AND ABBREVIATIONS

<b>Abi</b>	Abortive infection
<b>BIM</b>	Bacteriophage insensitive mutant
<b>cfu</b>	Colony forming unit
<b>CRISPR</b>	Clustered Regularly Interspaced Short Palindromic Repeat
<b>GHP</b>	Good Hygienic Practices
<b>GLP</b>	Good Laboratory Practices
<b>GRAS</b>	Generally Regarded As Safe
<b>HACCP</b>	Hazard Analysis Critical Control Point
<b>MAP</b>	Modified Atmosphere Packaging
<b>pfu</b>	Plaque-forming units
<b>QAC</b>	Quaternary ammonium compound
<b>QPS</b>	Qualified Presumption of Safety
<b>PCR</b>	Polymerase chain reaction
<b>R-M</b>	Restriction-modification
<b>RTE</b>	Ready-to-eat
<b>vgc</b>	Virulence gene cluster